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Band 15 nr 11

GERD BENDZ

A study of the chemistry of some
Marasmius species



ALMQVIST & WIKSELL / STOCKHOLM

1959

A STUDY OF
THE CHEMISTRY OF SOME
MARASMIUS SPECIES

INAUGURAL DISSERTATION

BY

GERD BENDZ

FIL. LIC., GOTL.

BY DUE PERMISSION OF THE FACULTY OF SCIENCE
OF THE UNIVERSITY OF UPPSALA, TO BE PUBLICLY DISCUSSED
IN THE LECTURE HALL OF THE INSTITUTE OF CHEMISTRY,
NOVEMBER 28, 1959, AT 10 A.M., FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY. IN ADDITION TO THIS
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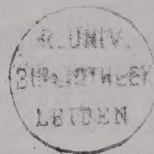
UPPSALA 1959

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A study of the chemistry of some *Marasmius* species

By GERD BENDZ

With 2 figures in the text



Introduction

Ever since the discovery of the medical potentialities of penicillin about twenty years ago a systematic world-wide search for new antibiotic substances in culture media of moulds and fungi, as well as in other materials of biological origin, has been carried on. These intensive investigations have revealed the existence of a large number of substances which, when being tested *in vitro*, exert an antibiotic effect on pathogenic bacteria. Unfortunately, however, these substances showed themselves in most cases to be unserviceable *in vivo*. The reasons for this were many; they were often found to exert a serious toxic effect or else were de-activated too rapidly. Only a very small number of the substances found to be active have therefore found a use in chemotherapy. The intensive search for new antibiotics has, however, brought with it an increased knowledge of the chemistry of fungi, in the culture media of which many new and unexpected substances have been found, and it has initiated further studies of their metabolism.

The present work will describe the investigation of active material in the culture media of some fungi belonging to the genus *Marasmius*, the isolation and identification of a polyacetylene (1), a methylnaphthaquinone (2) and an isocoumarin derivative (3) and treat some related problems arising in connection with the isolated substances (4, 5, 6). It will also describe the isolation of other active substances, of which some have been proved to be polyacetylenes. It has, however, not been possible to further purify these compounds or try to identify them owing to the small amount of material available.

During the search for new antibiotics Melin *et al.* (7) found in 1947 that several fungi belonging to the genus *Marasmius* produce antibiotic substances when cultivated under appropriate conditions. In order to investigate these antibiotics, a collaboration was started between the Institute of Physiological Botany, the Chemical Institute, University of Uppsala, and the State Bacteriological Laboratory, Stockholm, under the direction of Professor E. Melin, Professor A. Fredga and Professor G. Olin, and the present work is a part of that investigation. For several reasons the investigation was discontinued in 1952. Since, however, one of the substances produced by *Marasmius ramealis* proved to be active against *Mycobacterium tuberculosis* (8), the work with this substance, which has been named marasin, has been continued

since 1955, with long intervals owing to shortage of material. The culture media which made possible the final elucidation of the structure of marasin were kindly supplied by A.B. Astra, Södertälje.

Fungus material

The genus *Marasmius* is of the class of Basidiomycetes ("higher fungi") and belongs to the family of Agaricaceae. The species of *Marasmius* used for this investigation have been *Marasmius ramealis* (Bull. ex Fr.) Fr., *Marasmius graminum* (Lib.) Berk. & Br. and *Marasmius urens* (Bull. ex Fr.) Fr. (correct name: *Marasmius peronatus* (Bolt. ex Fr.) Fr.). These fungi, which were collected and isolated by Lindeberg (9), were usually grown as surface cultures upon a modification of the Czapek-Dox substrate. As it turned out that two of the fungi, *Marasmius ramealis* and *Marasmius urens*, increased their antibiotic production on the addition of citric acid, a solution of sodium citrate was added to their culture liquid about two weeks after inoculation with the fungi (10). In order to obtain marasin in larger quantities, several attempts were also made to cultivate *Marasmius ramealis* as submerged cultures in large flasks and tanks. In these experiments the same substrate as in the surface cultivations was used, but with the addition of 1 % of soy bean oil, in order to prevent foaming. The active material which the three fungi form was found in their culture media, and only in the case of *Marasmius graminum* did the dried and ground mycelium contain traces of active material.

Isolation of active material

Steam-volatile compounds

Two strains of *Marasmius ramealis* have been investigated and both form several steam-volatile substances. Different methods of isolating marasin, the active polyacetylene existing amongst them, have been worked out (1). The methods have in common that the active material is extracted from the culture liquid with the aid of ether and that the residue, after the ether has been removed in vacuum, is submitted to steam distillation under reduced pressure. The first strain of *Marasmius ramealis* which was investigated produced at least four steam-volatile substances. One of these, an isocoumarin derivative (3), could be separated from the other three by extraction with petroleum ether. The substances left in the aqueous distillate were all polyacetylenes, and with the aid of repeated counter-current distribution in fourteen funnels between carbon tetrachloride and water, marasin could be separated from the other two polyacetylenes. A partial separation of the two last-mentioned was obtained by means of chromatography on de-activated alumina (2, 11), the elution being performed with a mixture of petroleum ether and benzene. It is worthy of note that it was not possible to elute marasin from any kind of de-activated alumina (12), although this adsorbent is frequently used for separation of polyacetylenes. A possible explanation of this unexpected behaviour of marasin which contains an allenic system, an unusual feature among the naturally occurring polyacetylenes hitherto isolated, might be that the allene group forms some kind of complex with alumina.

The other strain of *Marasmius ramealis* which was investigated produced several,

closely related steam-volatile compounds. Marasin was separated from this mixture by distributing it between *cyclohexane* and water containing 20 % ethanol in a Craig apparatus with 200 tubes. When the ultraviolet absorption at 263 $m\mu$ of the different solutions were plotted against the corresponding tube number the distribution curve thus obtained indicated the presence of six fractions (Fig. 2, ref. 1). Under the experimental conditions used only the main component, marasin, was obtained in a pure form, whereas the other fractions contained mixtures of two or more substances. It was also possible to separate marasin from the other components in the aqueous distillate with the aid of chromatography on a moist mixture of activated charcoal and diatomaceous earth, the elution being performed with moist ether. The aqueous distillate from the culture media to which soy bean oil had been added contained a large amount of various compounds, of which marasin formed only a small part. With the aid of counter-current distribution between petroleum ether and water in a Craig apparatus with fifty tubes, followed by adsorption on a cellulose-powder column from pentane containing 5 % ethanol, the elution being made with the same solvent, spectrophotometrically pure marasin could be obtained in small quantities (1).

Marasmius graminum produces two quinonoid substances active against bacteria and, on account of their volatility with steam, these substances could be isolated from the filtered culture medium by submitting the latter to steam distillation under reduced pressure. After repeated steam distillation and recrystallisation from petroleum ether, the quinonoid mixture was obtained as red needles (m.p. 87–88°C). They were finally separated from each other by adsorption on de-activated alumina from ligroin. Elution with ethanol gave a yellow quinone and the subsequent elution with aqueous methanol containing a small amount of acetic acid gave a red quinone (2).

Non-volatile compounds

The greater part of the active, non-volatile material which the three fungi produce was extracted with ether from the culture media or from the aqueous solution left after steam distillation. These ethereal extracts were each divided into an acid and a neutral fraction by counter-current distribution between ether and a solution of either a phosphate buffer with pH slightly above 7 or 0.01 *M* sodium bicarbonate.

The acid fraction from the three fungi contained very small quantities of highly impure, active material and evaporation of the solvent always gave yellowish-brown gums. No definite substance could be isolated from it with the aid of counter-current distribution in funnels, chromatography or micro-sublimation. In order to trace the active material in the different fractions which were then obtained, their effect was tested against *Staphylococcus aureus* *H* by the cylinder cup method. To judge by the results obtained, the three fungi in all probability produce several active acid substances.

As regards the neutral fractions which have been obtained from *Marasmius ramealis* and *Marasmius graminum*, these also were highly impure and, to judge by the attempts made to purify them, they also contained several active compounds. Preliminary experiments carried out on the neutral fraction from *Marasmius graminum* indicate that at least one of the active substances in it is a naphthahydroquinone (2). With the aid of chromatography of an ethereal solution on de-activated alumina (12), the elution being performed with ether-benzene, one of the active substances formed by the first-used strain of *Marasmius ramealis* was isolated, although not in

a pure form. To judge by its absorption in ultraviolet, it is a polyacetylene compound.

The neutral fraction from *Marasmius urens* also contains one, possibly several, closely related, active polyacetylenes, which have been isolated from this fraction with the aid of repeated counter-current distribution in funnels between water and chloroform-carbon tetrachloride (1:1).

A common feature of the active material in the three culture liquids was that a small part of it was not soluble in any of the usual organic solvents. The inextractable character of this part of the active material might be due to a hydroxyl group present in one of the antibiotic substances forming a conjugate of some kind (13), soluble in water but insoluble in the common organic solvents. At least, this seems to be the case with the steam-volatile substance, marasin, produced by *Marasmius ramealis*. When, after the ether-soluble material had been removed by extraction, the culture liquid was submitted to steam distillation, the distillate contained a small amount of the volatile, active alcohol, marasin, even after several weeks of distillation. A similar increase of solvent-extractable material has been found to occur when the culture media from the fungi *Polyporus biformis* (14) and *Agrocybe dura* (15) were boiled and in both cases the polyacetylene compounds isolated have been proved to contain hydroxyl groups.

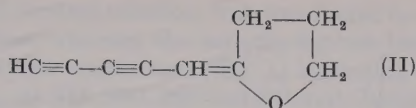
Polyacetylenes

As has been mentioned before, *Marasmius ramealis* produces several steam-volatile polyacetylenes. The main component, marasin, is a very unstable substance which has been identified as (-)-nona-3,4-diene-6,8-diyne-1-ol (I), the optical activity being due to the allene system (1).



The ultraviolet absorption spectrum of marasin, with λ_{max} at 237, 249.5, 263 and 278 μ , indicated the presence of an ene-diyne group and the infrared spectrum revealed that marasin contains acetylenic hydrogen (3280 cm^{-1}), disubstituted acetylene (2200 cm^{-1}), allene (1960 cm^{-1}) and alcoholic hydroxyl (3570 and near 1050 cm^{-1}). On hydrogenation of marasin with Pd/BaSO_4 ca. 5 moles of hydrogen were absorbed. The hydrogenation product was submitted to a gas chromatographic analysis, in which four different fractions were obtained. The main fraction was identified by means of its mass spectrum, which was identical with that of nonane-1-ol. The mass spectra of the other fractions proved them to be straight-chain, primary, unsaturated, C_9 alcohols. Since the hydrogenation product only consists of primary, straight-chain, C_9 alcohols, marasin must contain a straight chain of nine carbon atoms. This fact, combined with the infrared and ultraviolet absorption data, proves that the structure of marasin must be I.

One of the most characteristic properties of marasin is its instability towards aqueous alkali, in which the biological activity disappears. At the same time, a change takes place in the ultraviolet absorption; the typical four-finger spectrum of marasin is replaced by a quite new type of spectrum, indicating the presence of a new substance. This, which has the same empirical formula as marasin, has been called *iso-marasin* and has been identified as tetrahydro-2-(penta-2,4-diynylidene)-furan (II) (4).



The ultraviolet absorption spectrum of *isomarasin* with λ_{max} at 224, 266, 279.5 and 295 $m\mu$ having the same characteristic features as that of octa-2-methoxy-2-ene-4,6-diyne (16), indicated the presence of a diyne-enol ether. According to the infrared spectrum, *isomarasin* contains acetylenic hydrogen (3340 cm^{-1}) and disubstituted acetylene (2230 cm^{-1}), and the sharp bands at 1642, 1160 and 1036 cm^{-1} suggested the presence of an unsaturated ether. The hydrogenation product of *isomarasin* was submitted to gas chromatography. The chromatogram revealed the presence of nonane-1-ol and of another substance, which was identified as tetrahydro-2-pentylfuran by its mass spectrum being identical with that of a synthetic sample. The presence of tetrahydro-2-pentylfuran as a hydrogenation product and the spectrophotometrical evidences prove that the structure of *isomarasin* must be II. In order to ascertain that the presence of nonane-1-ol in the hydrogenation mixture is due to the cleavage of the tetrahydrofuran ring, a model experiment was carried out with tetrahydro-2-methylfuran (17). This substance was hydrogenated under the same experimental conditions as those used for *isomarasin* and the hydrogenation mixture was submitted to gas chromatography. The chromatogram revealed the presence of not only tetrahydro-2-methylfuran but also of pentane-1-ol, proving that under these conditions a fission of the tetrahydrofuran ring also occurs.

Marasin, which has a high optical activity due to the presence of the allene group, represents an unusual type of natural product. A few similar compounds have recently been discovered, *viz.*, the four substances in the nemotin series produced by three different Basidiomycetes (18, 19) and mycomycin produced by an Actinomycete (20). What is common to these compounds, besides their activity against *Mycobacterium tuberculosis*, is their transformation in alkaline solutions to new substances, whereby the allene group is lost. The activity against the above-mentioned bacteria seems mainly dependent on the presence of the allene group in conjugation with the acetylene groups, since the activity after the isomerisation is either greatly reduced or completely lost.

The aqueous distillate from the first-used strain of *Marasmius ramealis* contained, besides marasin, two other substances, which gave the same characteristic, four-finger spectra as marasin, the maxima being displaced only 1–4 $m\mu$ towards longer wave-lengths. These two substances, which, to judge from their ultraviolet absorption spectra seem to be polyacetylenes containing an ene-diyne group, could not be completely separated from each other. The results obtained when trying to separate them indicated, however, that, if a more effective method of separation had been used, these two substances would probably turn out to be a mixture of several different polyacetylenes. Owing to the similarity of their absorption spectra to that of marasin, they could not be detected in marasin-containing fractions, unless the latter were made alkaline and the very volatile *isomarasin* was distilled off; the ultraviolet spectrum of the remaining aqueous solution then indicated their presence or absence, as they did not undergo any noticeable change on the addition of alkali.

As has been previously mentioned, the steam-volatile compounds in the aqueous distillate from the last-used strain of *Marasmius ramealis* could be divided up into, besides marasin, five different fractions, the main component of at least four of which

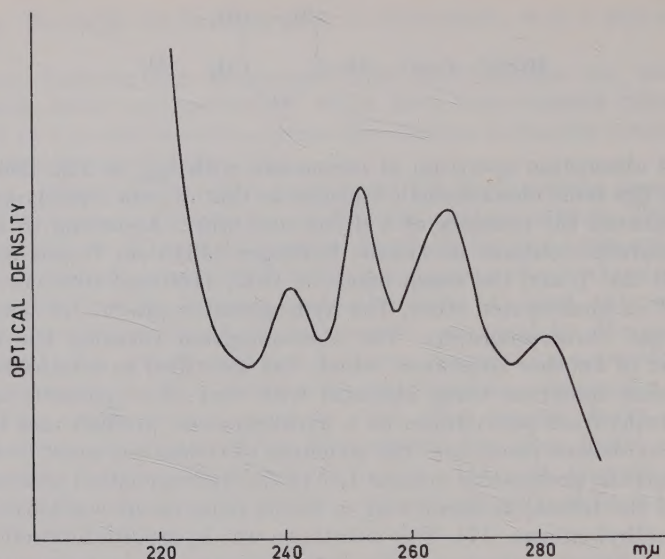


Fig. 1. Ultraviolet absorption spectrum of a polyacetylene, isolated from the culture liquid of *Marasmius urens*.

might be a polyacetylene containing an ene-diyne chromophore. According to their ultraviolet spectra, none of these four polyacetylenes had been obtained in a pure state and only one of them did not change when treated with alkali. Whether any of these polyacetylenes was identical with any of those formed by the first strain of *Marasmius ramealis* is impossible to decide, since the last-mentioned were not completely separated from each other.

The neutral, ether-soluble fraction from *Marasmius ramealis* contained also at least one polyacetylene. To judge from its ultraviolet spectrum, $\lambda_{\max}$ at 250, 265 and 282 $m\mu$, this substance contains an ene-diyne group. The presence of a hydroxyl group or ester group could also be demonstrated. It is, however, not out of the question that this fraction, as well as the acid fraction, contains a mixture of polyacetylenes, since several of the attempts made to isolate them indicated such a mixture.

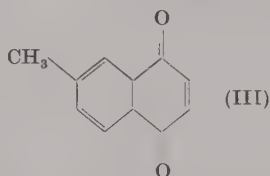
From the results obtained so far it is evident that *Marasmius ramealis* produces a series of closely related polyacetylenes and, probably, the number of such compounds is larger than what was forthcoming in this investigation. It seems as if such series of closely related polyacetylenes are not unusual phenomena in culture media from Basidiomycetes. Thus, for example, seven polyacetylenes have recently been isolated from the culture medium of *Coprinus quadrididus* (21) and a mixture of at least seventeen such compounds from *Polyporus anthracophilus* (22).

It has earlier been found (23) that *Marasmius urens* also produces at least one antibiotic polyacetylene, which has been isolated from the neutral, ether-soluble fraction. After purification it has been obtained as a colourless precipitate turning violet to black in the air. It did not contain halogen, sulphur or nitrogen, but a hydroxyl group or ester group, and formed a photosensitive precipitate with silver nitrate, possibly indicating the presence of a free ethynyl grouping. Furthermore,

it was stable in acid and neutral solution, but was found to undergo an irreversible change in alkaline solution, whereby the activity against bacteria disappeared. The substance gave in ultraviolet a spectrum (Fig. 1) indicating the presence of an enediyne chromophore ($\lambda_{\max}$ at 241, 252, 265 and 281 $m\mu$). Judging from the appearance of the spectrum, the substance isolated was probably still contaminated with other compounds. The instability in alkaline solution resembled that of the acetylenic allenes. As, however, the presence of an allene group is best shown with the aid of the infrared spectrum and an infrared spectrophotometer was not at this time available, it was impossible to decide whether the compound isolated contained an allene group or not.

Naphthalene derivatives

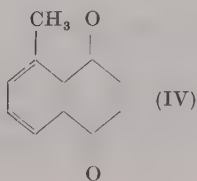
The yellow quinone produced by *Marasmius graminum* has been identified as 6-methyl-1,4-naphthaquinone (III) (2)



Elementary analysis of a condensation product with 2,4-dinitrophenylhydrazine pointed to a methylnaphthalene derivative. The α -quinonoid structure was established by catalytic hydrogenation to a leuco-compound, by reductive acetylation to a diacetyl-dihydro-derivative, by certain colour reactions (24) and by the ultraviolet absorption spectrum which showed a great similarity to those of 1,4-naphthaquinone and 2-methyl-1,4-naphthaquinone. The position of the methyl group was determined by oxidative degradation to trimellitic acid, the anhydride of which was identified by its mixed melting point with a synthetic sample. The structure of the isolated compound was finally proved by the synthesis of III. Diene condensation of p-benzoquinone with isoprene yielded an "isoprene-quinone", which was oxidised with sodium dichromate to 6-methyl-1,4-naphthaquinone.

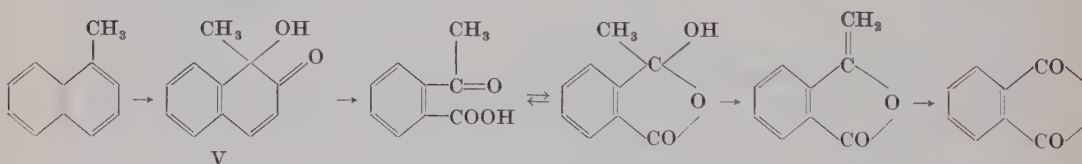
Since, after the chromatographic separation, only a very small amount of the red quinone was obtained, no trials were made to identify it. Some of its properties indicated, however, a hydroxynaphthaquinone structure.

Ever since the discovery of Boissevain (25) that 2-methyl-1,4-naphthaquinone inhibited the growth of tubercle bacilli a large number of 1,4-naphthaquinone derivatives with different substituents in the 2 and 3 position has been investigated as regards their activity against this as well as against other bacteria. 1,4-Naphthaquinones with a methyl group in the benzenoid nucleus had not been submitted to a similar investigation, and in order to compare the biological activity of the three possible methyl-1,4-naphthaquinones, 5-methyl-1,4-naphthaquinone (IV) was also synthesised (5)



Two 5-methyl-1,4-naphthaquinones with different melting points were described in the relevant literature. One of these, prepared by oxidation of 5-methyl-1,4-naphthalenediamin (26) and melting at 121–122°C was proved by another synthetic route, mentioned below, to be the right substance. The other 5-methyl-1,4-naphthaquinone reported was obtained by oxidation of commercial 1-methylnaphthalene and melted at 102–103°C (27). When the last synthesis was repeated, it was found that the naphthaquinone obtained was the 2-methyl derivative which was derived from 2-methylnaphthalene contaminating the 1-methylnaphthalene used. However, if the latter compound was carefully purified, it yielded on oxidation about 2 % of a quinone melting at 121–122°C. This quinone was identified as 5-methyl-1,4-naphthaquinone synthesised in the following way. By diene condensation of piperylene and p-benzoquinone a "piperylene-quinone" was formed. This was isomerised to 5,8-dihydro-5-methyl-1,4-naphthahydroquinone which on oxidation yielded IV melting at 121–122°C. This melting point was not depressed on admixture with the quinone obtained on oxidation of purified 1-methylnaphthalene.

1-Methylnaphthalene contaminated with only about 14 % of the 2-methyl derivative yielded on oxidation with chromic acid in acetic acid mainly 2-methyl-1,4-naphthaquinone, whereas purified 1-methylnaphthalene under the same conditions only gave about 2 % of quinone. Consequently, the oxidation reaction must in the latter case proceed in another way than that of the oxidation of 2-methylnaphthalene. An investigation of the reaction mixture obtained on oxidation of purified 1-methylnaphthalene indicated that it seems to be degraded mainly via 2-acetobenzoic acid to phthalic acid (6). It is possible that the first attack by the oxidising agent is made at the position 1 and 2 in the methylnaphthalene, since 1-methyl-1,2-naphthaquinol (V) was found in the reaction mixture, and that it is further degraded to 2-acetobenzoic acid. The presence of 3-methylenephthalide as an oxidation product was proved to be due to 2-acetobenzoic acid being dehydrated in the acid solution. It was also found that 3-methylenephthalide is spontaneously oxidised to phthalic anhydride. The main degradation of 1-methylnaphthalene should thus proceed in the following way:

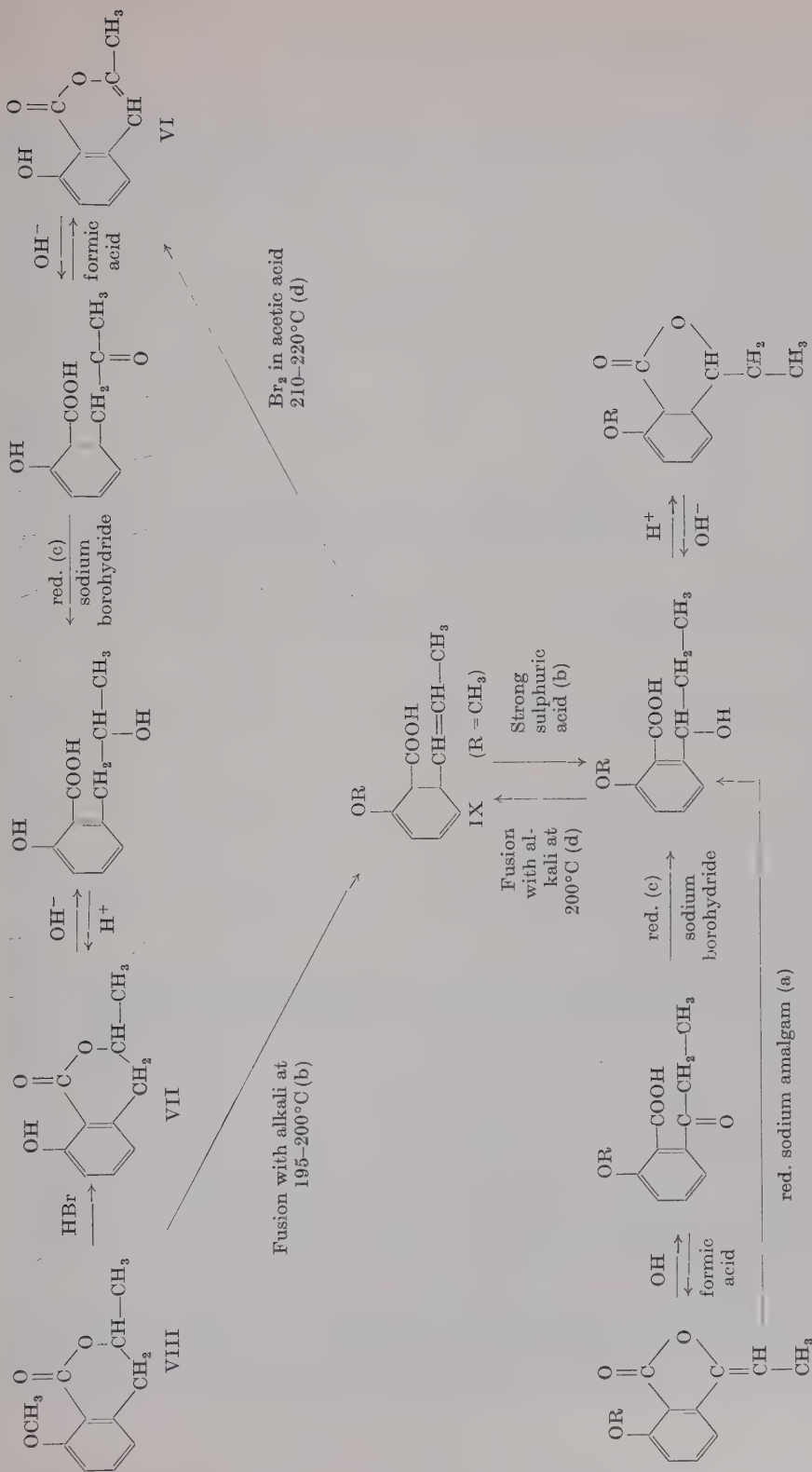


1-Naphthoic acid was isolated from the reaction mixture in an amount of 5–10 % of the methylnaphthalene used. According to a preliminary investigation, this acid is also attacked by the oxidising agent yielding phthalic acid. A possible explanation of the presence of this unexpected oxidation product might be that 1-naphthoic acid, like several other organic acids (28), is capable of forming chromium complexes.

Isocoumarin derivative

Among the steam-volatile compounds produced by the first-used strain of *Marasmius ramealis* was one substance which was easily recognised by the intensive blue-violet colour developed with ferric chloride. After purification by means of steam

Chart I.



X (R = H)

^a Ref. 29; ^b Ref. 32; ^c Ref. 33; ^d Ref. 34.

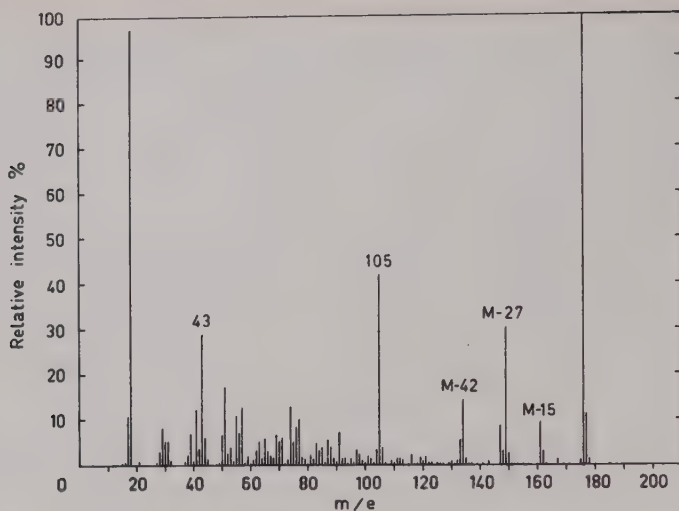


Fig. 2. Mass spectrum of 8-hydroxy-3-methylisocoumarin.

distillation, the substance was obtained as a colourless precipitate, which, after being recrystallised from aqueous methanol, yielded colourless needles melting at 99–100°C. This substance has been identified as 8-hydroxy-3-methylisocoumarin (VI Chart I) (3).

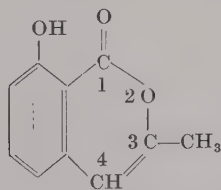
The colour developed with ferric chloride suggested a salicylic acid type of structure and the presence of an hydroxyl group was confirmed by the formation of a methyl ether with dimethyl sulphate and of a benzoate with benzoyl chloride. The elementary analysis of the isolated substance and of its benzoate indicated a composition of $C_{10}H_8O_3$. The presence of a lactone ring in the position 1 and 2 to the hydroxyl group was demonstrated by the formation of an acid when the substance was treated with hot alkali and by degradation of the methyl ether to 3-methoxyphthalic acid. A shift of the carbonyl band towards higher frequencies was observed in the infrared spectra of the methyl ether and the benzoate when comparing these spectra with that of the phenolic compound. This indicates that some kind of intramolecular hydrogen bond exists between the carbonyl and hydroxyl groups in the latter substance.

These facts suggested that the phenolic compound was either a phthalide or an isocoumarin derivative. Chart I shows the relationships between the relevant isocoumarins and phthalides. It was hardly possible that the phenolic compound had the structure X, since it was recovered unchanged after treatment with sodium amalgam (29). Furthermore, the infrared and ultraviolet absorption spectra of the unknown substance, after comparing them with the spectra of known isocoumarins and phthalides, suggested that the most probable structure was that of an isocoumarin derivative.

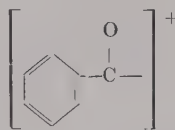
A laevo-rotatory substance called *mellein* has been isolated from the fungi *Aspergillus melleus* (30, 31) and *Aspergillus ochraceus* (32) and identified as 3,4-dihydro-8-hydroxy-3-methylisocoumarin (VII), the structure having been confirmed by the synthesis of the corresponding racemic methyl ether (VIII) (33). This substance was

used as starting material for the synthesis of the phenolic compound isolated which was performed via the acid IX (Chart I). The structure was further confirmed by converting the phenolic compound to the corresponding dihydroderivative by the route VI→VII (Chart I). The identity of the synthesised substances were in both cases proved by mixed melting points and by the infrared and ultraviolet absorption spectra.

A trial was also made to establish the structure of the phenolic compound by studying its mass spectrum (Fig. 2). Since the only similar mass spectra investigated are those of four- and five-membered aliphatic lactones (35), it was, however, not possible to interpret the different peaks in the spectrum of the *isocoumarin* derivative, until its structure was determined. The largest peak is situated at $m/e=176$ corresponding to the ionised unfragmented molecule, suggesting that, like unsaturated aliphatic five-membered lactones, the stability of the parent molecule ions is great. The peak at $m/e=161$ is obviously due to the rupture of the side chain bond. Since α -angelicalactone has a peak at $m/e=43$ and the fragment $C_2H_3O^+$ is known to be of high stability, it is possible, that the peaks at $m/e=134$ and 43 in this spectrum might be due to a splitting of the bonds between the atoms in the position 1 and 2, 3 and 4 (XI) followed by rearrangement, although this involves a breaking of a double bond. A possible interpretation of the peak at $m/e=149$ is a splitting of the bonds between the atoms 2 and 3, 3 and 4 (XI), and that the higher intensity of this peak might be due to the fragment having ring closed to a phthalide ring. The 105 peak might perhaps be due to the fragment XII.



(XI)



(XII)

8-Hydroxy-3-methylisocoumarin was not active against *Staphylococcus aureus*, whereas mellein is reported to exhibit a weak antibacterial activity against this bacteria (31).

Antibacterial activity *in vitro*

Marasin. The antibacterial activity was determined by means of two-fold dilutions in infusion broth. Each tube containing 2 ml was inoculated with a loop of a 24-hour broth culture diluted 1:1000 of the organism being tested. Another test method used was to incorporate two-fold dilutions in a series of agar plates. The test organism in appropriate dilution was inoculated on the agar surface. The lowest concentration preventing visible growth after 24 hours' incubation at 37°C was in each case determined. For *Mycobacterium tuberculosis* the agent was incorporated in solid Dubos' medium. The end points were read after 10 days at 37°C. The following results were obtained (36):

Test organism	No. of strains tested	Range of minimum inhibitory concentration ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i>	26	1.0-4.0
<i>Staphylococcus albus</i>	3	1.0-2.0
<i>Streptococcus pyogenes</i>	2	> 32
<i>Streptococcus group D</i>	4	> 32
<i>Diplococcus pneumoniae</i>	2	> 32
<i>Escherichia coli</i>	9	16-> 16
<i>Pseudomonas aeruginosa</i>	1	> 16
<i>Salmonella typhi</i>	1	8
<i>Salmonella paratyphi</i>	1	16
<i>Vibrio cholerae</i>	2	4-8
<i>Mycobacterium tuberculosis</i>	20	0.25-1.0
<i>Canadida species</i>	6	8-32

Substance produced by Marasmius urens. The antibacterial activity of this antibiotic which is probably identical with the polyacetylene found in the culture medium of this fungus, was determined by incorporating a solution containing the polyacetylene, the concentration of which was unknown, in a series of agar plates as described above. It was then found that the antibiotic inhibited the growth of the following organisms (36)

Staphylococcus aureus (8 strains tested)
Streptococcus group D
Escherichia coli
Pseudomonas aeruginosa
Salmonella typhi
Bacillus subtilis
Mycobacterium tuberculosis (7 strains tested)

Methylnaphthaquinones. The activity of the three methylnaphthaquinones was tested by diluting the agent in broth as described above. The results were as follows (36)

Test organism	6-methyl- -1,4-naphtha- quinone	5-methyl- -1,4-naphtha- quinone	2-methyl- -1,4-naphtha- quinone
<i>Staphylococcus aureus</i> , 209 P	1.6	1.6	3.1
<i>Streptococcus hemolyticus</i>	200	100	6.2
<i>Streptococcus viridans</i>	> 200	100	12.5
<i>Bacillus subtilis</i>	> 200	25	12.5
<i>Escherichia coli</i>	> 200	> 200	200
<i>Salmonella typhi</i>	> 200	> 200	200
<i>Pseudomonas aeruginosa</i>	> 200	> 200	> 200

Some biosynthetic aspects

During the last five years it has been found that polyacetylenes are produced by a variety of fungi when grown on synthetic or semi-synthetic media (13, 37, 38). Since most of the fungal polyacetylenes, until recently isolated, are produced by

organisms which first attracted interest because of their antibiotic-producing activity, the study of their distribution has not yet been systematic. The polyacetylenes occur, however, so frequently that they can no longer be considered as rare exceptions among fungal metabolites; investigations of their occurrence in fungal cultures, as well as of their metabolism, are therefore in progress in some laboratories.

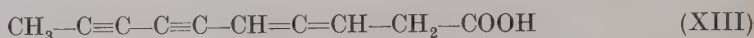
Bu'Lock and Leadbeater (39) have studied the conversion of glucose into polyacetylenes by the Basidiomycete B 841, which produces the nemotin compounds. Their evidence seems to indicate that the polyacetylenes which this fungus produces are no by-products, but that their formation is "an alternative metabolic pathway for some intermediate product of 'normal' glucose breakdown". Bu'Lock and Gregory (40) have also been able to prove that the carbon chain of nemotinic acid is built up from acetate units. By adding ($1-^{14}\text{C}$) acetate to the culture liquid of B 841 and degrading the isolated nemotinic acid, they found that the radioactivity was confined to all the odd-numbered carbon atoms. They suggested that nemotinic acid with 11 carbon atoms is built up from six acetate units by head-to-tail linkage and that the terminal methyl group (which remains in the accompanying C_{12} odyssic acid) is eliminated by oxidation followed by decarboxylation. Further evidence that the polyacetylenes with an odd number of carbon atoms, which all contain a free ethynyl group, might arise from compounds with an even number of carbon atoms by ω -oxidation and decarboxylation is provided by the isolation of the many polyacetylenes produced by *Polyporus anthracophilus* (22). Most of these are derivatives of *n*-decane with terminal methyl groups or products (alcohols or acids) of their ω -oxidation.

According to Bu'Lock and Gregory (40), it is possible that the formation of fungal polyacetylenes represents close analogies to the biosynthesis of simple fatty acids and that the triple bond arises in some way from $-\text{CO}-\text{CH}_2$ -groups produced in the repeated condensation of acetyl-coenzyme A. It has, however, not yet been possible to state whether the unsaturation is introduced before the carbon chain is built up or after or how the triple bond is formed. One possible formation of the triple bond might be the dehydration of the enol form of the $-\text{CO}-\text{CH}_2$ -group, enolphosphate perhaps being an intermediate (41). The reverse reaction, that is, the hydration of a triple bond, has been demonstrated to occur in certain strains of *Enterobacteriaceae* (42) and every fermentative reaction ought to be reversible.

It seems as if the polyacetylenes which occur most frequently (not only among the Basidiomycetes but also among the higher plants) are those derived from *n*-decane and varying in the extent of unsaturation and degree of oxidation at either end of the chain. On the other hand, various acetylenic acids containing the C_{18} straight chain of stearic acid have been isolated from seed oils and in most of these the unsaturation is situated between C_9 and C_{18} in the chain. Since one of these acids, erythro-*gonic* acid, isolated from the nuts of an African tree, *Ongokea klaineana*, is very easily split, forming a C_{10} polyacetylene probably derived from the C_9 to the C_{18} part of the acid (43), it might be possible that at least the C_{10} polyacetylenes are formed by a similar splitting of unsaturated C_{18} acids.

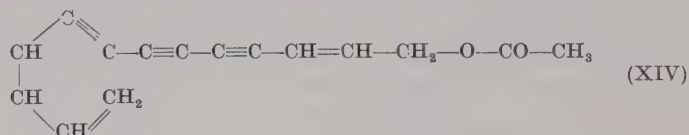
The nutritional effects upon the antibiotic production of *Marasmius ramealis*, as well as of *Marasmius urens*, were investigated by Öblom (10, 44) before it was proved that at least part of the antibiotic action of these two fungi is due to the presence of polyacetylenes in their culture liquids. She found that the two fungi produced the antibiotic substances even when grown on a medium containing glucose as the only source of organic matter. This suggests that also in this case the carbon source of the polyacetylenes is glucose and that they, like nemotinic acid, are formed from

acetate units. It might be possible that the C_9 compound, marasin (I), in analogy with nemotinic acid, is formed from a C_{10} acid having the formula XIII by subsequent oxidation, decarboxylation and reduction.

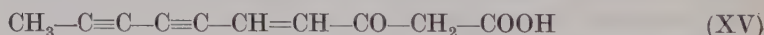


Since the first strain of *Marasmius ramealis* investigated contained, besides marasin, a steam-volatile C_{10} compound, 8-hydroxy-3-methylisocoumarin (VI), the two compounds might be derived from a common precursor. This may be a β -polyketo acid, which is built up from five acetate units, and which, according to the hypothesis of Birch (45), can either ring-close to the isocoumarin derivative or in some way dehydrate, forming the acid XIII, the suggested precursor of marasin.

However, it has been found that, *in vitro*, some acetylene compounds isomerise to furan derivatives (46) and in connection with the isolation of 1-acetoxy-*n*-trideca-2, 10,12-triene-4,6,8-triyn from *Carlina vulgaris* Sørensen and Sørensen (47) suggested that, in analogy with the above-mentioned isomerisation, a benzene ring might be formed by curling up of the diene-yne tail of XIV



This suggestion has been further substantiated by the isolation from the *Daedalea juniperina* culture of anisaldehyde and the acetylenic thiophen aldehyde junipal. According to Birkinshaw and Chaplen (48), these two substances might be formed from a common straight-chain acetylenic precursor which might either carbon-to-carbon ring-close to form the benzene ring or incorporate an external sulphur atom to form the thiophen ring. Should such a ring-closing reaction to benzene derivatives be biogenetically possible, a common precursor to the two substances isolated from *Marasmius ramealis* could be an acetylenic acid with the formula XV

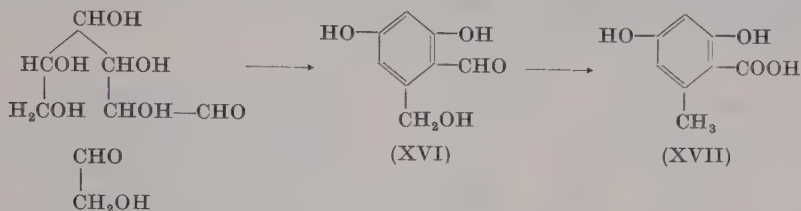


This might isomerise to 8-hydroxy-3-methylisocoumarin or form, possibly by reduction of the keto group followed by dehydration, the acid XIII, from which marasin might be derived as postulated above.

Only a few isocoumarin derivatives have been found in nature and of these the bitter principle in carrots, 3,4-dihydro-6-methoxy-8-hydroxy-3-methylisocoumarin (49) is obviously derived from orsellinic acid (XVII). In analogy with this acid it might be formed by cyclisation of a β -polyketo acid in accordance with the acetate hypothesis of Birch (45). A similar synthetic route has also been proposed for mellein (VII) (50), isolated from two species of *Aspergillus*. This hypothesis has been substantiated by the isolation from moulds of some compounds as 6-hydroxy-2-methylbenzoic acid (51, 52, 53) and 6-carboxy-3,5-dihydroxybenzylmethylketone (54, 55), related to mellein as well as to orsellinic acid. Furthermore, by means of marked carbon atoms 6-hydroxy-2-methylbenzoic acid has been proved to be built up of acetate units (56). Some evidence seems also to be available that 3,5-diketohexanoic acid might be an intermediate (45). An alternative formation of mellein from a

polyacetylene precursor as proposed above for 8-hydroxy-3-methylisocoumarin is probably not possible since no polyacetylenes have hitherto been isolated from moulds.

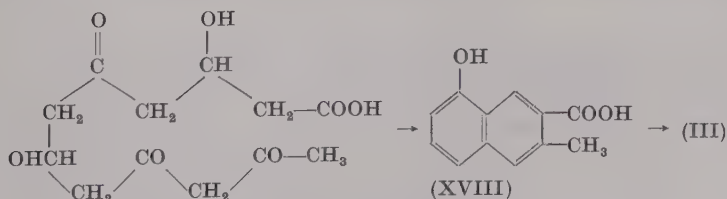
In this connection it must be pointed out, however, that Seshadri *et al.* have elaborated another theory of biosynthesis of mould products (57). They suggest that the C_8 compound (XVI) is derived by aldol-type condensation of a hexose and a biose and that orsellinic acid (XVII) arises therefrom by side-chain oxidation and reduction.



According to the authors various known benzenoid mould products (as mellein and the two acids mentioned above) might be formed from these "orsellinic units" by known processes of oxidation and reduction and condensation with other chemical units. Evidently, this biogenetic scheme could also be applied to 8-hydroxy-3-methylisocoumarin. In such a case, however, it seems impossible that the isocoumarin derivative and the polyacetylenic acid (XIII) could be derived from a common precursor.

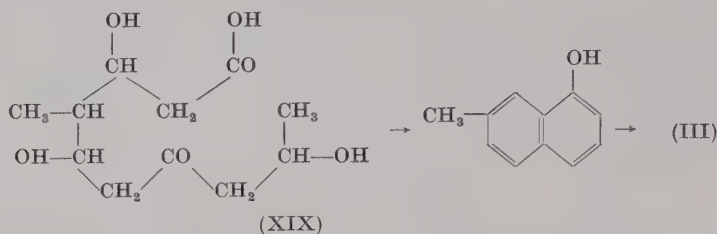
Some substituted naphthaquinones have been isolated from fungal material, *viz.* the hydroxynaphthaquinones produced by several species of *Fusarium* (58, 59, 60) and 2,5,7-trihydroxy-1,4-naphthaquinone, named flaviolin, isolated from *Aspergillus citricus* (61). In no case has the structure of the *Fusarium* naphthaquinones been completely established but it seems as if they all contain hydroxyl groups in the benzenoid nucleus and alkyl-containing side-chains in the quinonoid one. Birch and Donovan (50) have put forward formulae for these quinones that are consistent with the acetate hypothesis and also in the same way postulated the formula of flaviolin. The latter has been confirmed by the synthesis of the quinone (62), giving further evidence of the usefulness of the acetate hypothesis.

6-Methyl-1,4-naphthaquinone isolated from *Marasmius graminum* differs from the other fungal naphthaquinones in that it does not contain any hydroxyl group and has the alkyl substituent in the benzenoid nucleus. It might be formed according to the acetate hypothesis by the head-to-tail linkage of six acetate units forming a methylhydroxynaphthoic acid XVIII which might decarboxylate and oxidise to the quinone III.



Since all naturally occurring naphthaquinones hitherto isolated, with the exception of III and oxydroseron (7-methyl-5,6,8-trihydroxy-1,4-naphthaquinone) and

its deoxyderivative (63), have the alkyl-containing side-chain in the quinonoid nucleus, a somewhat different biosynthetic route than that postulated above might also be possible. According to Woodward (64), not only acetate units but also propionate units might form β -polyketo acids and some fatty acids isolated from bacteria have been proved to have a structure clearly indicating a precursor built up of acetate and propionate units. According to this hypothesis, a possible precursor of 6-methyl-1,4-naphthaquinone might be the β -polyketo acid XIX built up of one propionate and four acetate units. This acid might after ring-closure and oxidation form the quinone III.



SUMMARY

The three fungi investigated, *Marasmius ramealis*, *Marasmius urens* and *Marasmius graminum* have been found to produce several acid as well as neutral antibiotics.

One of the active substances produced by *Marasmius ramealis* has been identified as (-)-nona-3,4-diene-6,8-diyne-1-ol. This fungus also produces several other closely related polyacetylenes and one isocoumarin derivative, identified as 8-hydroxy-3-methylisocoumarin.

One polyacetylene, active against bacteria, has been isolated from the culture medium of *Marasmius urens* and, judging from its ultraviolet absorption spectrum, it also contains an ene-diyne chromophore.

Two antibiotic naphthaquinones have been isolated from the culture medium of *Marasmius graminum*. One of these has been identified as 6-methyl-1,4-naphthaquinone.

The antibacterial activity of the isolated compounds is recorded and several problems arising in connection with the isolated substances have been treated.

ACKNOWLEDGEMENTS

To my teacher, Professor Arne Fredga, I wish to express my sincere gratitude for his never-failing interest in this work, for many stimulating discussions and for all the encouragement I have received.

To Professor Elias Melin, the initiator of this work, I am also greatly indebted for his kind interest in the present investigation and for the material prepared at his institute.

I also wish to thank Professor N. A. Sörensen, Trondheim, and Professor E. R. H. Jones, Oxford, for their interest in the work with the polyacetylenes and for many helpful suggestions.

My thanks are also due to my colleagues, Dr. Karin Öblom and Docent Gösta Wallmark, for their friendly collaboration and for many fruitful discussions in connection with this investigation.

I am also very indebted to Docent Gösta Wallmark for the determination of the antibacterial activities of the different substances related in this paper, to Dr. A. Bengtsson for the gas chromatographic analysis and to Ingeniör R. Ryhage for taking the mass spectrum.

The help of Mrs. M. Englund and Mrs. B. Flemström with the preparation of my manuscripts is greatly appreciated.

Grants from the *Swedish Natural Science Research Council*, from *A.B. Astra*, Södertälje, and from the *Swedish Anti-Tuberculosis League* are gratefully acknowledged.

The English in this paper has been revised by Mr. N. Tomkinson.

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Tryckt den 4 november 1959

Uppsala 1959. Almqvist & Wiksells Boktryckeri AB

